Kidney International, Vol. 58 (2000), pp. 1641–1651

H^+-ATPase activity on unilateral ureteral obstruction: Interaction of endogenous nitric oxide and angiotensin II

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Background. A number of cytokines, vasoactive compounds, chemoattractant molecules, and growth factors are up-regulated in obstruction. Following the onset of ureteral obstruction, angiotensin II production is rapidly stimulated. Cytokine-induced expression of inducible nitric oxide synthase (iNOS) has been reported in primary cultures of inner medullary collecting duct (IMCD) cells. We found that the defective urinary acidification in unilateral ureteral obstruction (UUO) includes an intensive decrease in bafilomycin-sensitive H^+-ATPase activity in microdissected IMCD segments.

Methods. To investigate the interaction between endogenous nitric oxide and angiotensin II on H^+-ATPase activity, we used microdissected IMCD segments of unilaterally obstructed, contralateral, and control kidneys to measure the bafilomycin-sensitive ATPase activity and nitric oxide synthase (NOS) activity. The generated NO was also evaluated.

Results. Preincubation of obstructed IMCD segments in the presence of a competitive inhibitor of NOS, N^G-nitro-L-arginine methyl ester (L-NAME) 1 mmol/L, and in the presence of a specific inhibitor of calcium/calmodulin-independent NOS (iNOS), aminoguanidine 1 mmol/L, each for 60 minutes, significantly increased bafilomycin-sensitive H^+-ATPase. A greater increase on iNOS activity (fmol [^{3}H] citrulline/min/μg protein) and a lesser increase in calcium/calmodulin-dependent NOS activity (cNOS) were observed in the obstructed renal medulla. This inhibitory effect of obstruction was abolished when IMCDs were incubated with 10^{-5} to 10^{-8} mol/L losartan. Decreasing doses of the angiotensin II type 1 (AT_1) receptor inhibitor caused an increase in bafilomycin-sensitive H^+-ATPase, with a maximum increase at 10^{-8} mol/L losartan. A decrease on iNOS activity was demonstrated in the obstructed renal medulla incubated with losartan in concentrations of 10^{-5} to 10^{-8} mol/L, the same losartan concentrations that showed recovery of vacuolar H^+-ATPase activity. Similarly, a decrease on the generation of NO after incubation with losartan 10^{-5} to 10^{-8} mol/L was shown.

Conclusion. From these results, we suggest that endogenous NO increased by iNOS is involved in the inhibition of H^+-ATPase activity in obstructed IMCD segments. The recovery of H^+-ATPase activity in IMCD of obstructed kidneys induced by losartan may be related to a decrease of inducible NOS activity.

Key words: inducible nitric oxide synthase, losartan, hemodynamics, fibrosis, renal interstitium, tubulointerstitium, inflammation.

Received for publication November 5, 1999
and in revised form March 29, 2000
Accepted for publication April 28, 2000

Chronic unilateral ureteral obstruction (UUO) is a well-established model of experimental renal injury [1]. UUO results in changes in renal hemodynamics [2], acidification duct defect [3], infiltration of the kidney by macrophages [4], and subsequently fibrosis of the tubulointerstitium [5].

The invasion of the renal interstitium by macrophages and T lymphocytes in rats coincides with a decline in renal hemodynamic parameters [6]. Vasoactive compounds such as angiotensin II, cytokines, and growth factors are up-regulated in obstruction. Angiotensin II production is rapidly stimulated following the onset of ureteral obstruction [7]. Most of the biological effects of angiotensin are transduced by angiotensin II type 1 (AT_1) receptor. Expression of AT_1 receptor mRNA in the distal tubule includes the cortical and medullary collecting duct segments [8].

Angiotensin II, in turn, up-regulates the expression of other factors, including tumor necrosis factor-α (TNF-α), osteopontin, and nuclear factor-κB [9].

In obstruction, up-regulation of TNF-α expression, a proinflammatory peptide produced by monocytes/macrophages and resident cells, has been reported in tubular renal cells [10]. TNF-α binding to its receptor activates signals of transduction pathways that result in the expression of a variety of transcription factors, other cytokines, growth factors, cell adhesion molecules, and mediators of inflammatory processes [11].

Exposure to TNF-α induces the expression of an inducible isoform of nitric oxide synthase (NOS) in the inner medullary collecting duct cells (IMCDs) [12].

The constitutive neuroneal isozyme of NOS has been localized in the macula densa cells and IMCDs [13].

The expression of inducible NOS (iNOS) mRNA in IMCDs microdissected from the normal rat has been recently demonstrated by reverse transcription-polymerase chain reaction [14].

IMCD segments play an important role in renal sodium, water, and acid excretion, and thay are often the site of injury in inflammatory diseases of the kidney.

Studies on H^+ secretion in nephron segments in the post-obstructed kidney have established that the collecting duct is the site of the acidification duct defect [15].

We have previously demonstrated an intense inhibition of bafilomycin-sensitive H^+-ATPase in IMCD segments of unilateral obstructed kidneys [16].

Several important actions of NO in the kidney have been demonstrated, including regulation of glomerular hemodynamics and sodium and water excretion [17], as well as participation in both immune-mediated [18] and hypoxia-reoxygenation–induced renal injury [19].

Evidence that nitric oxide inhibits proton pump activity and may thus play a role in the regulation of proton and bicarbonate transport has been shown in cortical collecting duct (CCD) segments of control rats [20].

Preliminary studies by Weiner et al have provided evidence for an effect of angiotensin II on acid-base transport in the CCD segments of the rabbit [21]. In support of this suggestion, involvement of angiotensin II in the modulation of an intercalated cell function at CCD segments has been reported by Tojo, Tisher, and Madsen [22].

The present protocol was performed to study whether endogenous NO is involved in the proton ATPase inhibition of IMCDs microdissected from unilateral ureteral obstructed rats, and to demonstrate the interaction between angiotensin II and NOS on the enzyme activity in unilateral obstruction.

METHODS

Animal model of unilateral urinary obstruction

Urinary tract obstruction of the left kidney of female Wistar Kyoto rats (180 to 200 g) was performed as follows. Animals were first anesthetized with ether.

Using an aseptic technique, a midline abdominal incision was performed; the left ureter was completely ligated with silk at the union between the pelvis and proximal ureter. The abdominal incision was closed with sutures, the rats were allowed to awaken, and were given free access to water and food. A sham group underwent a fictitious surgery. A nephrectomy of the left kidney was performed 24 hours after the operation. Rats were considered to have a successful ureteral obstruction when ureteral dilation was >2 mm at 24 hours.

Microdissection of tubule segments

The kidneys were perfused from the abdominal aorta with 10 mL of cold dissection solution containing in (mmol/L) 100 NaCl, 3.7 ClMg, 2 $CaCl_2$, 66.7 NH_4Cl, 50 imidazol, and 5.5 glucose to rinse away all of the blood.

The left and right kidneys of the experimental group and the left kidneys of the control group were removed. The kidneys were cut along the corticopapillary axis. Slices of 1 mm in thickness were placed into the dissection solution at 4°C without collagenase, and were immediately microdissected by hand with the aid of fine stainless steel needles under a stereomicroscope (×10 to ×40). IMCD segments measuring between 1 and 1.5 mm were dissected from the proximal end at the corticomedullary boundary, including the outer third of the inner medulla. Between 16 and 20 initial IMCD segments were dissected from each animal. The length of each segment was measured under the stereomicroscope using an eyepiece micrometer.

Assay for H^+-ATPase activity

The ATPase activity was measured as described [23] with minor modifications. Briefly, the microdissected tubule segments were rinsed in a cold dissection solution, suspended in the same solution, and transferred to 0.5 mL Eppendorf tubes (1 tubule segment/4 μL). The samples were frozen until use. After thawing, each sample was incubated in 37°C for 15 minutes in the presence of 1 mmol/L adenosine 5′-triphosphate (ATP).

The reaction was stopped by immersing the Eppendorf tube into a boiling water bath for three minutes. The samples were microcentrifuged, and the free inorganic phosphate generated during the reaction was measured in the supernatant using the malachite-green colorimetric method, as follows. The supernatant was diluted with dissection solution up to 200 μL and mixed with 1 mL of the malachite-green reagent (0.02%) malachite green oxalate, 0.03% $Na_2MO_4 \cdot 2\ H_2O$, 0.05% Triton X-100, 0.7 mol/L (HCl). An addition of 0.1 mL of 33% sodium citrate solution to every 1 mL of this mixture was made; this step stabilizes the color reaction and makes it insensitive to nascent phosphate. After incubating for 30 minutes at room temperature, the optical density was read at 600 nm. The standard curve was performed by incubating 200 μL of the known phosphate concentration in solution with 1 mL of malachite-green reagent. The inorganic phosphate concentration present in the samples was determined from a linear plot of the standards.

The ATPase activity was expressed as the picomole of inorganic phosphate per minute per millimeter of tubule length.

Bafilomycin-sensitive H^+-ATPase activity

The H^+-ATPase activity was measured as the bafilomycin-sensitive ATPase activity. The microdissected tubule segments were preincubated for 40 minutes at 37°C with the specific inhibitors ouabain (4 μmol/L), omeprazole (100 μmol/L), and bafilomycin (10 nmol/L) before incubating with ATP (discussed previously in this article).

The bafilomycin-sensitive H^+-ATPase activity was defined as the difference between the activity in the pres-

ence of the three inhibitors and in the presence of ouabain-omeprazole without bafilomycin. Bafilomycin is a specific inhibitor for the vacuolar H^+-ATPase [24].

In our assay, we used 10 nmol/L bafilomycin to inhibit the ATPase activity in IMCDs. This bafilomycin concentration was chosen from the lineal regression correlation of increasing bafilomycin doses that were related to the maximal inhibition of H^+-ATPase activity tested in control tubules. For each animal, the H^+-ATPase activity was determined in triplicate samples for different experimental conditions: the presence of Na^+,K^+-ATPase and H^+,K^+-ATPase without and with inhibitor of H^+-ATP ase. The mean value was considered as a single point. The final result represents the means ± SEM of 10 animals. In each experiment, blanks were also run in triplicate to correct the spontaneous hydrolysis of ATP with time.

Protocol I

In all the experiments, intact microdissected tubules from the obstructed kidney (OK), contralateral kidney (ClK), and control kidney (CK) were preincubated in assay buffer containing the various compounds to be tested. After preincubation, the tubules were transferred to fresh assay buffer and subjected to freezing and thawing before determination of H^+-ATPase activity, as described previously in this article.

Effect of L-NAME on H^+-ATPase activity. To examine whether inhibition of H^+-ATPase activity is caused by induction of endogenous NO in intercalated cells of IMCD, incubation of the microdissected segments of OKs, ClKs, and CKs was performed for 60 minutes in the presence of 1 mmol/L N^G-nitro-L-arginine methyl ester (L-NAME), a competitive inhibitor of NOS [25], before measuring the bafilomycin-sensitive H^+-ATPase activity.

Effect of aminoguanidine on H^+-ATPase activity. In another series of experiments, we examined the effect of aminoguanidine, a relatively selective inhibitor of iNOS [26], on bafilomycin-sensitive H^+-ATPase activity. Microdissected IMCD tubule segments of OKs, ClKs, and CKs were incubated for 60 minutes in the presence of aminoguanidine 1 mmol/L, before the measurement of bafilomycin-sensitive H^+-ATPase activity.

Effect of AT_1 receptor inhibition on H+-ATPase activity. We evaluated the involvement of angiotensin II through its AT_1 receptor on the inhibition of H^+-ATPase activity, using a specific inhibitor of the AT_1 receptor, losartan [27].

The individual microdissected IMCD segments of OKs, ClKs, and CKs were incubated in an assay buffer containing losartan at concentrations ranging from 10^{-5} to 10^{-8} mol/L for 60 minutes at 37°C before the determination of H^+-ATPase activity.

Protocol II

Bilateral nephrectomy from obstructed and control ether anesthetized rats was performed. Slices from the kidney were done, and the renal medulla was removed under a stereomicroscope. After weighing, the medulla was immediately frozen on dry ice. Later, the whole tissue was homogenized using a glass homogenator (Lightnin Model Mixer-Volts 100; Mixing Equipment Co. Inc., Rochester, New York, USA) in a solution (10 mg tissue/100 μL solution) containing N-hydroxyethyl-piperazine-N′-2-ethanesulfonic acid (HEPES)-Tris 10 mmol/L, Sacarose 0.32 mol/L, dithiothreitol (DTT) 1 mmol/L, soybean tripsin inhibitor 1 mg/100 mL, and Trasylol 2.5 μg/mL to pH 7.40. The same homogenate of each kidney was used for both the measurement of nitrite release and the NOS enzyme assay.

All chemicals were purchased from Sigma (St. Louis, MO, USA) unless otherwise noted.

Determination of nitrite release in homogenates from renal medulla. We measured the release of nitrite from kidney using a previously described method [28].

Homogenates from each renal medulla from OKs, ClKs, and CKs were incubated with 10 mmol/L L-arginine in a buffer (pH 7.40) containing 25 mmol/L HEPES, 140 mmol/L NaCl, 5.4 mmol/L KCl, 1.8 mmol/L $CaCl_2$, 1 mmol/L $MgCl_2$ and 5 mmol/L glucose at 37°C for 24 hours.

After centrifugation at 6400 r.p.m. during 20 minutes, the supernatants were used for the assay of NO_2 production, and the amount of NO_2^- was corrected by means of the protein amount, measured according to the Bradford method using bovine serum albumin as a standard (Bio-Rad, Richmond, CA, USA) [29].

Nitrite was measured by a spectrophotometer at a 540 nm wavelength using the Griess reaction [30]. The NO present was expressed as picomoles of nitrite generated per milligram of proteins per minute.

Aliquots of renal medulla homogenates from CKs ($N = 8$) were incubated with 10 mmol/L L-arginine and with the NOS inhibitor, 20 mmol/L L-NAME, for 24 hours to ensure that NO was produced in the homogenates of renal medulla.

Determination of NOS activity. Nitric oxide synthase activity was quantitated by measuring the conversion of L-[^{3}H] arginine to L-[^{3}H] citrulline, using the presence of saturating concentrations of the enzyme's cofactors as previously described [31] with minor modifications.

Homogenates from renal medulla were centrifugated at 3000 r.p.m. at 4°C for 10 minutes. A 40 μL aliquot of the each supernantant fraction was incubated with 3 mmol/L $CaCl_2$, 1 mmol/L NADPH, 25 μmol/L FAD, 1.25 μg/mL calmodulin, 10 μmol/L tetrahydrobiopterin, and [^{3}H] arginine (approximately 300,000 cpm, 6.8 Ci/mL) in 20 mmol/L HEPES buffer (reaction buffer), pH 7.40, at 37°C for 30 minutes. Calcium/calmodulin-independent NOS activity was measured by using the same buffer and cofactors, without calcium and calmodulin, after the addition of 0.5 mmol/L ethylenediaminetetraacetic acid (EDTA). Parallel reactions were analyzed in the presence of L-NAME 20 mmol/L, an inhibitor of

the inducible and constitutive isoforms of NO synthase, in the renal medulla of CKs. The reaction was stopped by the addition of the L-NAME–20 mmol/L HEPES buffer (100 μL) at 4°C. The total volume (210 μL) was applied to a Dowex 50 W-X8, 100 to 200 mesh column (volume 0.6 mL) that had been pre-equilibrated with 20 mmol/L HEPES (7.40) and saturated with 20 mmol/L of cold citrulline. [^{3}H] citrulline was eluted with 200 μL of deionized water, and radioactivity was quantitated by scintillation counting (Wallac, LK Beta Rack, Finland). Blanks included 50 μL of homogenate buffer plus 60 μL of reaction buffer without or with calcium/calmodulin for measuring iNOS and cNOS, respectively. The results are expressed as fmol [^{3}H] citrulline/μg protein/min incubation.

Protein concentrations were measured according to Bradford by using a Bio-Rad reagent [29]. Bovine serum albumin was used as standard (1 μg/mL).

Experimental protocols

Effect of losartan on nitrite release in renal medulla homogenates. We measured the nitrite release from renal medulla homogenates in OKs, ClKs, and CKs after the tissues were incubated in the presence of losartan at concentrations ranging from 10^{-5} to 10^{-8} mol/L, for five hours at 37°C.

Simultaneously, an aliquot from renal papilla homogenates from CKs was incubated with 20 mmol/L L-NAME for five hours to ensure that NO was produced in the kidney homogenates.

Effect of losartan on NOS activity in renal medulla homogenates. To evaluate the interaction between angiotensin II and endogenous nitric oxide, preincubation of renal medulla homogenates in the presence of an inhibitor of the AT_1 receptor, losartan, on concentrations ranging from 10^{-5} to 10^{-8} mol/L for five hours was performed. After incubation with losartan, the calcium/calmodulin-dependent NOS activity (cNOS) and calcium/calmodulin-independent NOS activity (iNOS) were measured in the medulla homogenates from OKs, ClKs, and CKs.

Statistical analysis

The results were assessed by one-way analysis of variance for comparisons between groups. Significance of differences was estimated by Bonferroni's test.

Student's test was used to compare the means when the experimental design consisted of two samples. Statistical significance was assessed by Student's impaired *t*-test. A $P < 0.05$ was considered to be significant. Results are given as means ± SEM.

RESULTS

Effect of endogenous NO on H^+-ATPase activity in obstruction

The compromise of H^+-ATPase activity on obstruction was evaluated by means of the bafilomycin-sensitive ATPase activity.

In IMCD of OKs, bafilomycin-sensitive H^+-ATPase activity was significantly decreased in comparison to the enzyme activity in microdissected IMCD of ClKs ($P < 0.01$) and CKs ($P < 0.01$; Fig. 1).

To determine whether endogenous NO was involved in the inhibition of bafilomycin-sensitive H^+-ATPase activity present in unilateral obstruction, the effect of L-NAME, a specific inhibitor of cNOS and iNOS, was studied. Incubation of IMCD of OK with 1 mmol/L L-NAME for 60 minutes showed a significant increase on bafilomycin-sensitive H^+-ATPase activity (18.09 ± 1.21 vs. 40.53 ± 1.96 pmol inorganic phosphate/min/mm; $P < 0.01$). After 60 minutes of incubation with L-NAME 1 mmol/L, no difference was observed in the H^+-ATPase activity of ClKs (69.73 ± 1.80 vs. 77.77 ± 2.20 pmol inorganic phosphate/min/mm) and CKs (61.18 ± 1.67 vs. 63.20 ± 2.45 pmol inorganic phosphate/min/mm; Fig. 1).

We also examined the effect of an inhibitor of inducible NOS, aminoguanidine, on bafilomycin-sensitive H^+-ATPase activity. Incubation of IMCD segments with aminoguanidine 1 mmol/L for 60 minutes was associated with a significant increase of H^+-ATPase activity on OKs (from 18.09 ± 1.21 to 45.33 ± 2.42 pmol inorganic phosphate/min/mm; $P < 0.01$).

After 60 minutes of incubation with aminoguanidine 1 mmol/L, no significant increase was observed on the enzyme activity in ClKs, 69.73 ± 1.80 versus 77.90 ± 4.07, and CK, 61.18 ± 1.67 versus 62.54 ± 1.81 pmol inorganic phosphate/min/mm (Fig. 1).

Effect of angiotensin II on H^+-ATPase activity in obstruction

To determine the participation of angiotensin II on the inhibition of bafilomycin-sensitive H^+-ATPase activity in obstruction, we examined the effect of an angiotensin II type 1 (AT_1) receptor inhibitor, losartan, at different concentrations on individual IMCD segments.

The effect of incubation of individual IMCD segments with losartan at concentrations ranging from 10^{-5} to 10^{-8} mol/L for 60 minutes at 37°C on bafilomycin-sensitive H^+-ATPase activity is illustrated in Figure 2.

With decreasing concentrations of losartan, there was a gradual increase in the recovery of H^+-ATPase activity. The strongest recovery of the enzyme activity was demonstrated at 10^{-8} mol/L of losartan.

In ClKs, no statistical differences were observed after the incubation with losartan at concentrations from 10^{-5} to 10^{-8} mol/L: 69.73 ± 1.80 versus 72.66 ± 4.63 (10^{-5} mol/L), 72.63 ± 3.29 (10^{-6} mol/L), 73.75 ± 4.42 (10^{-7} mol/L), and 75.66 ± 1.90 (10^{-8} mol/L). There was no effect of losartan concentrations from 10^{-5} to 10^{-8} mol/L on control IMCD proton ATPase activity: 61.18 ± 1.67 vs. 54.40 ± 1.35 (10^{-5} mol/L), 56.16 ± 1 (10^{-6} mol/L), 53 ± 1.20 (10^{-7} mol/L), and 56 ± 2 (10^{-8} mol/L).

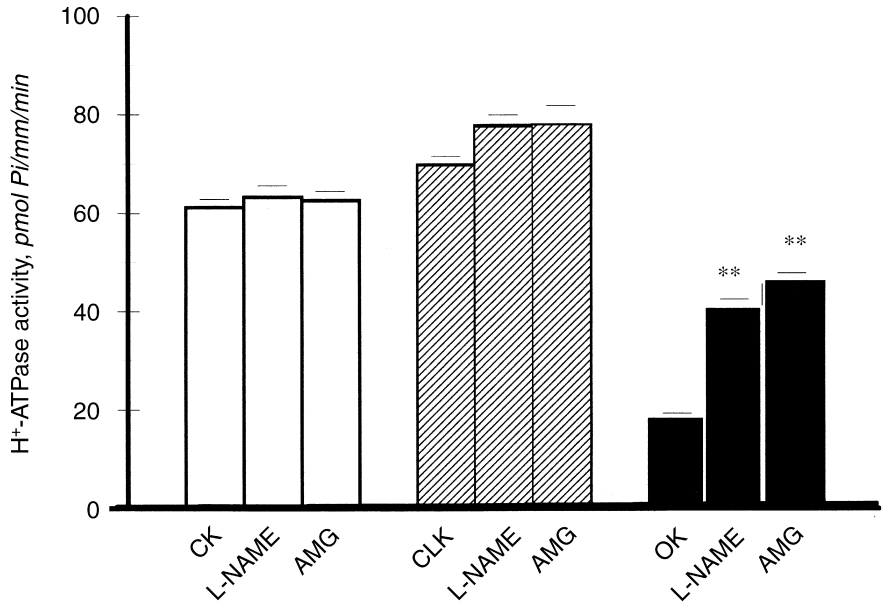


Fig. 1. Bafilomycin-sensitive ATPase activity in rat inner medullary collecting duct (IMCD) segments after 24 hours of unilateral ureteral obstruction. Symbols are: (□) control ($N = 10$); (▨) contralateral ($N = 10$); (■) obstructed kidney (OK; $N = 10$). Effect of L-NAME, a competetive inhibitor of nitric oxide synthase (NOS), on bafilomycin-sensitive ATPase activity in rat IMCD. Tubules were incubated with L-NAME 1 mmol/L for 60 minutes at 37°C. A significant increase on bafilomycin-sensitive H^+-ATPase activity in IMCD of OK is shown ($^{**}P < 0.01$). Effect of 1 mmol/L aminoguanidine (AMG), an inhibitor of inducible NOS (iNOS), on bafilomycin-sensitive ATPase activity in rat IMCD. Tubules were incubated with aminoguanidine 1 mmol/L for 60 minutes at 37°C. We found a significant increase in bafilomycin-sensitive H^+-ATPase activity in IMCD of OK ($^{**}P < 0.01$). Values are mean ± SEM.

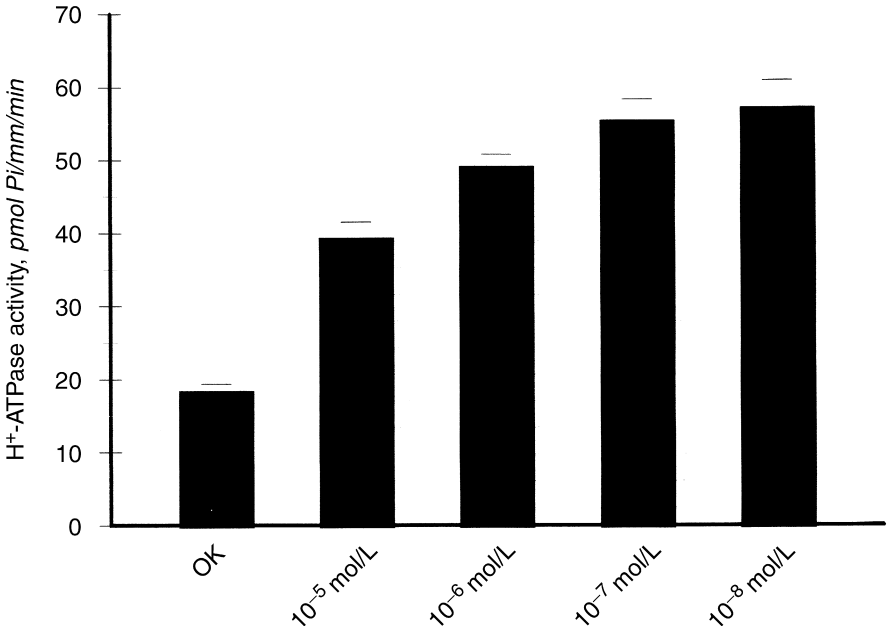


Fig. 2. Effect of losartan at concentrations of 10^{-5} to 10^{-8} mol/L on unilateral obstruction-mediated inhibition of H^+-ATPase activity in rat IMCD. Tubules were incubated with losartan for 60 minutes at 37°C. Decreasing doses of losartan increased H^+-ATPase activity.

NOS activity on renal medulla tissue in obstruction

Nitric oxide synthase activity was quantitated by measuring the conversion of L-[^{3}H] arginine to L-[^{3}H] citrulline. When homogenates from renal medulla tissue of OKs were incubated in the presence of saturating concentrations of the enzyme cofactors, without calcium/calmodulin, a significant increase in activity of calcium/calmodulin-independent NOS (iNOS) related to ClKs (0.89 ± 0.04 vs. 0.47 ± 0.02 fmol [^{3}H] citrulline/min/μg protein; $P < 0.01$) and CKs (0.89 ± 0.04 vs. 0.41 ± 0.02 fmol [^{3}H] citrulline/min/μg protein; $P < 0.01$) was demonstrated (Fig. 3).

There was a slight but significant increase in calcium/calmodulin-dependent NOS activity in the medulla of the obstructed rat kidneys when compared with medulla of contralateral rat kidneys (0.60 ± 0.03 vs. 0.41 ± 0.02, $P < 0.05$) and control (0.60 ± 0.03 vs. 0.26 ± 0.01 fmol [^{3}H] citrulline/min/μg protein; $P < 0.05$; Fig. 3).

The interaction between angiotensin II and NOS activity was evaluated in obstruction. Preincubation of renal

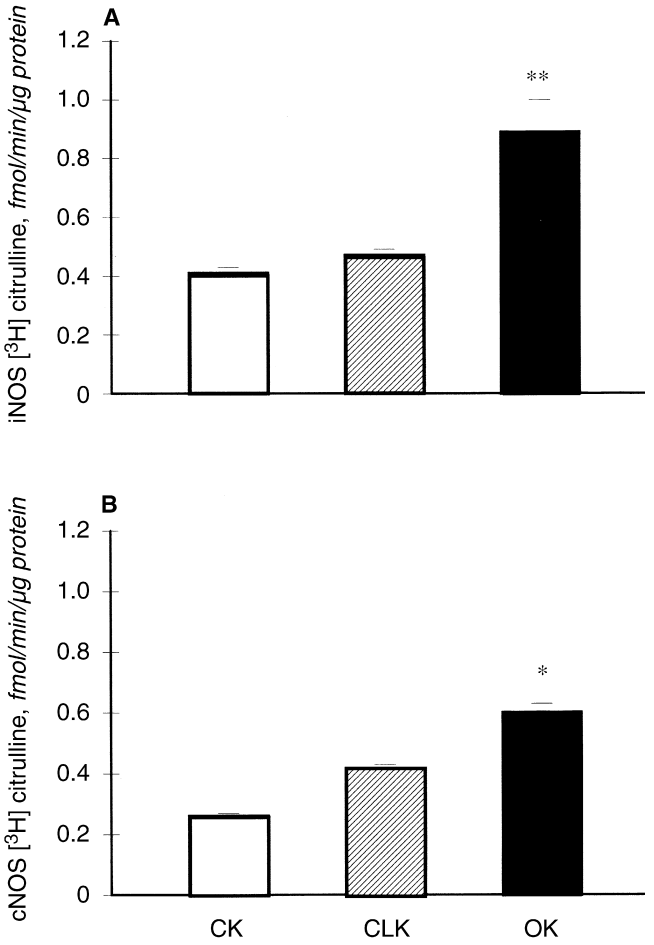


Fig. 3. Nitric oxide synthase activity in rat renal papilla homogenates after 24 hours of unilateral obstruction from (□) control (CK; $N = 22$), (▨) contralateral (ClK; $N = 22$), and (■) obstructed (OK; $N = 22$) kidneys. (*A*) Calcium/calmodulin-independent nitric oxide synthase activity (iNOS). $^{**}P < 0.01$, OK vs. ClK and OK vs. CK. (*B*) Calcium/calmodulin-dependent NO activity (cNOS). $^{*}P < 0.05$, OK vs. ClK and OK vs. CK. Values are mean ± SEM.

medulla tissue of OKs for five hours with concentrations of 10^{-5} to 10^{-8} mol/L losartan induced a sharp decrease of calcium/calmodulin-independent NOS activity (Fig. 4).

A lesser decrease of calcium/calmodulin-dependent NOS activity was observed in the renal medulla of OKs after incubation of 10^{-5} to 10^{-8} mol/L losartan for five hours (Fig. 4).

Measurement of calcium/calmodulin-independent and calcium/calmodulin-dependent NOS activity on homogenates of the renal medulla of ClKs showed a small decrease in NOS activity. No differences in the homogenates of the renal medulla of CKs were observed in calcium/calmodulin-independent and -dependent NOS activity (Fig. 4).

The addition of 20 mmol/L L-NAME caused a significant decrease in the generation of [^{3}H] citrulline in the renal medulla of CK (0.41 ± 0.02 vs. 0.19 ± 0.02; $P < 0.05$), indicating that [^{3}H] citrulline formation closely reflected renal NO synthesis.

Determination of nitrite

Consistent with the NOS data, measurement of nitrite generated from the homogenates of renal medulla from the 24-hour obstructed kidneys was significantly higher compared with the same tissue of ClKs (38.80 ± 2.27 vs. 25.67 ± 0.95, $P < 0.01$) and CKs (38.80 ± 2.27 vs. 28.3 ± 0.67 nmol NO_2 generated/min/mg protein, $P < 0.01$; Fig. 5).

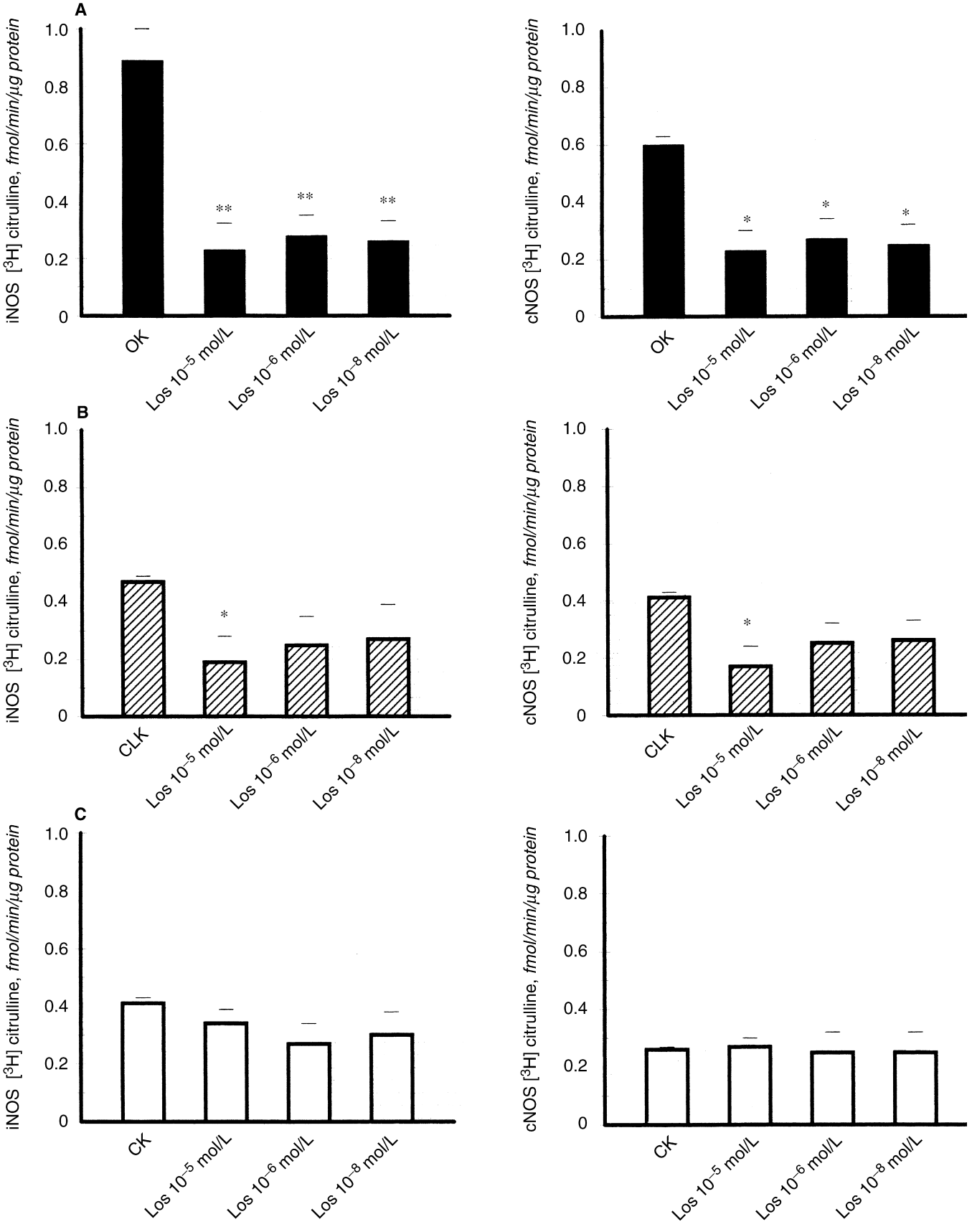
Measurement of nitrite generated from the papilla of OKs incubated in the presence of losartan in doses of 10^{-5} to 10^{-8} mol/L demonstrated a decrease on nitrite excretion with decreasing doses of losartan in a dose-dependent manner [38.80 ± 2.27 vs. 32.01 ± 0.81 nmol NO_2 generated/min/mg protein (10^{-5} mol/L), 18.90 ± 1.56 nmol NO_2 generated/min/mg protein (10^{-6} mol/L)]. A maximum decrease of nitrite excretion was observed in homogenates of OKs with a losartan dose of 10^{-8} mol/L (38.80 ± 2.27 vs. 9.14 ± 0.57 nmol NO_2 generated/min/mg protein).

We observed that the smaller the concentration of losartan, the higher the decrease in the nitrite generation in the homogenate of the renal obstructed medulla. A small decrease of nitrite excretion was observed on homogenates from renal medulla of ClKs (25.67 ± 0.95 vs. 19 ± 1.10 nmol NO_2 generated/min/mg protein, $P < 0.059$) after losartan 10^{-5} mol/L administration. No differences were observed on homogenates from ClKs after losartan 10^{-6} mol/L (25.67 ± 0.95 vs. 22.90 ± 1.52) and 10^{-8} mol/L (25.67 ± 0.95 vs. 23.88 ± 1.60 nmol NO_2 generated/min/mg protein). Renal medulla from controls showed no differences after incubation for five hours in the presence of losartan at concentrations from 10^{-5} to 10^{-8} mol/L [28.30 ± 0.67 vs. 26.06 ± 0.97 (10^{-5} mol/L), 24.13 ± 1.78 (10^{-6} mol/L), and 21.86 ± 1.17 nmol NO_2 generated/min/mg protein (10^{-8} mol/L); Fig. 5].

DISCUSSION

The results of this study demonstrate that endogenous nitric oxide is involved in the H^+-ATPase inhibition in

Fig. 4. Effect of losartan 10^5 to 10^8 mol/L on nitric oxide synthase activity in rat renal medulla. Renal medulla homogenates were incubated with losartan for five hours at 37°C. (*A*) There was an intensive decrease of calcium/calmodulin-independent NOS activity in OKs ($^{**}P < 0.01$) and a decrease of calcium/calmodulin-dependent NOS activity in OKs ($N = 7$; $^{*}P < 0.05$) after incubation with losartan. (*B*) Calcium/calmodulin-independent and -dependent NOS activity in ClKs, ($N = 7$), after incubation with losartan. (*C*) Calcium/calmodulin-independent and -dependent NOS activity in CKs ($N = 7$), after incubation with losartan. Values are mean ± SEM.


A
iNOS [3H] citrulline, fmol/min/μg protein
cNOS [3H] citrulline, fmol/min/μg protein
1.0
0.8
0.6
0.4
0.2
0
OK
Los 10–5 mol/L
Los 10–6 mol/L
Los 10–8 mol/L
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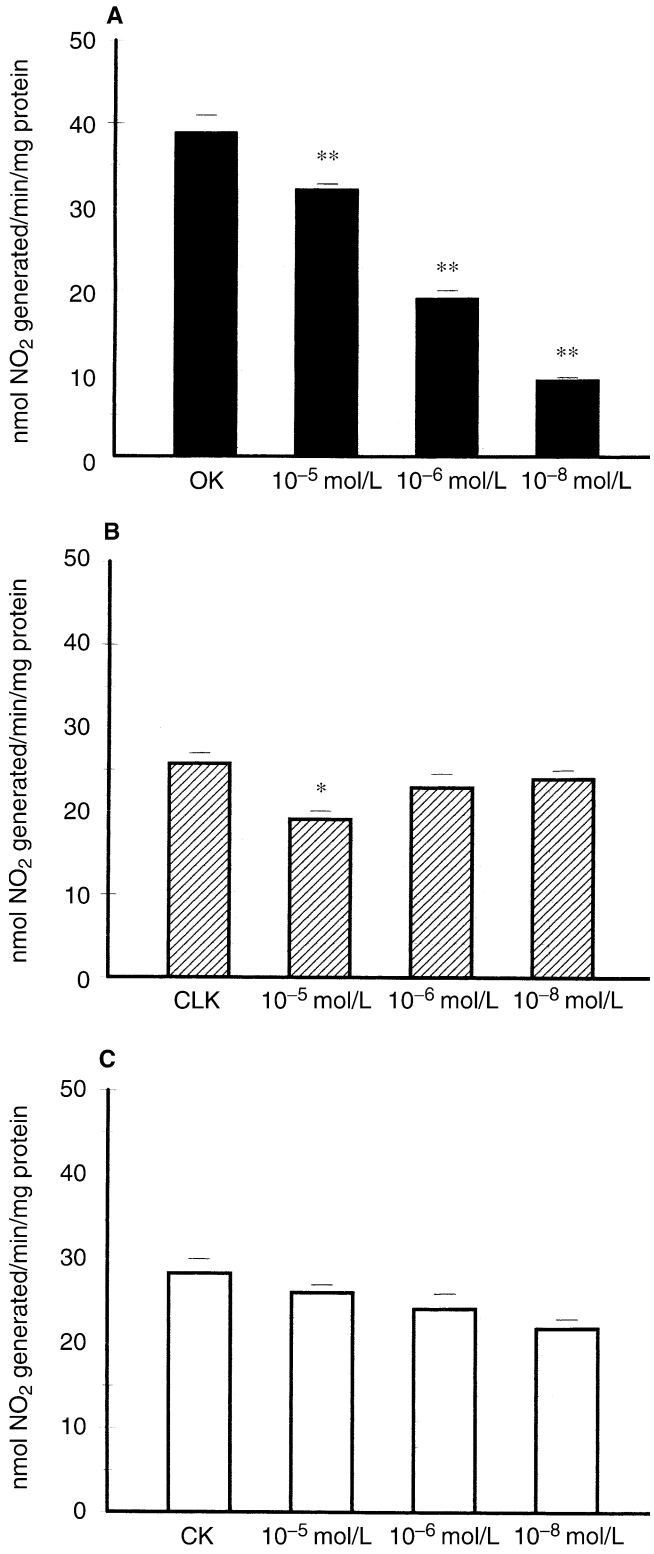

IMCD segments microdissected from unilateral OKs. The intensive inhibition of bafilomycin-sensitive H^+-ATPase activity in IMCD segments obstructed for 24 hours was reversed after incubation of the duct segments in the presence of a competitive inhibitor of NOS, L-NAME. A significant increase in bafilomycin-sensitive H^+-ATPase in obstructed IMCD was shown after incubation in the presence of L-NAME.

There was no effect of L-NAME on the bafilomycin-sensitive H^+-ATPase activity in the isolated IMCD from the contralateral and CKs.

Because bafilomycin is considered to be a specific inhibitor of the vacuolar H^+-ATPase at the concentration used in our experiments, the nitric oxide generated during obstruction may be involved in the regulation of the vacuolar proton ATPase in the intercalated cells.

High levels of the electrogenic vacuolar H^+-ATPase have been found in the intercalated cells of the connecting and collecting tubules, in outer medullary collecting ducts (OMCD), and in intercalated cells of IMCD. The presence of A type intercalated cells has been demonstrated on the initial segments of the IMCD tubules, the duct segments used in our present study [32].

Immunocytochemical studies have shown that in unilateral obstruction, morphological changes in H^+-ATPase included the appearance of a gap in apical H^+-ATPase staining of the intercalated cells, suggesting an impairment in the cytoskeletal translocation of the enzyme in the inner medulla [33]. We have previously shown an intensive decrease in the bafilomycin-sensitive H^+-ATPase activity in medullary collecting duct segments with lesser degree of the proton activity in the CCD segments after 24 hours of unilateral ureteral obstruction [16].

In our present study, a significant recovery of bafilomycin-sensitive H^+-ATPase in obstructed IMCD segments was also induced by aminoguanidine, a selective inhibitor of the inducible isoform of NO synthase, with a Ki value that is 32 to 52 times less for iNOS than for constitutive NOS [34].

A slight increase of the enzyme activity was observed in obstructed IMCD with aminoguanidine in comparison to the incubation of the same injured IMCD in the presence of L-NAME. According to this latest finding, evidence of an intensive increase of calcium/calmodulin-independent NOS activity (iNOS) in homogenates from

Fig. 5. Effect of 10^5 to 10^8 mol/L losartan on the generation of endogenous nitric oxide in rat renal medulla. Renal medulla homogenates were incubated with losartan for five hours at 37°C. (*A*) An intensive dose-dependent decrease on the nitrite generation in obstructed kidneys (OKs; $N = 7$), after incubation with losartan (**$P < 0.01$). (*B*) Nitrite generation in contralateral kidneys (CLKs; $N = 7$), after incubation with losartan (*$P < 0.05$). (*C*) Nitrite generation in control kidneys (CKs $N = 7$) after incubation with losartan. Values are mean ± SEM.

the renal papilla of OKs was shown in our study, whereas a lower activity of iNOS was observed in the renal papilla of ClKs and CKs.

In this experimental model of unilateral ureteral obstruction, a number of cytokines, vasoactive substances such as angiotensin II, chemoattractant molecules, and growth factors, are up-regulated. These inflammatory cytokines have proved to activate inducible NOS gene transcription in IMCDs [12]. Recent evidence also suggests that iNOS may be constitutively expressed in normal rats.

Since basal iNOS gene expression is high in medullary thick ascending limb and IMCD segments that normally function in a relatively hypoxic environment, the recent discovery that iNOS is a hypoxia-inducible gene provides at least one mechanism that may account for the constitutive expression of iNOS in these segments [35]. Results of an increased iNOS compared with cNOS activity in renal medulla of CKs in our study suggests a higher basal activity of iNOS.

The two major constitutive NOS isozymes expressed in IMCD segments—nNOS and eNOS—exhibit a strict dependency on intracellular calcium/calmodulin.

By using reverse transcription-polymerase chain (RT-PCR) reaction in microdissected renal segments, Terada et al observed nNOS mRNA principally in the inner medullary collecting ducts [13]. The eNOS mRNA and its protein have been reported in renal cortex and medulla of the rat [36]. Although they are classified as constitutive enzymes, the expression of nNOS and eNOS is regulated by specific physiological and pathophysiological stimuli, including increases in local vascular resistance that enhances shear stress [37] and hypoxia [38]. In unilateral obstruction, renal hemodynamics include a selective increase in renal vascular resistance and a decrease in the glomerular filtration rate of the ipsilaterally obstructed kidney.

Even though the calcium/calmodulin-dependent nitric oxide activity (cNOS) in the OK was higher than that of the ClK and CK, iNOS activity in the OK was significantly increased when compared with the cNOS activity in this impaired kidney.

Thus, our results suggest the presence of an early inducible isoform of NOS activation in this tubule segment.

We also showed that there was an increased generation of nitrite in the renal medulla of the OK.

L-NAME, an inhibitor of both L-arginine transport and NOS activity, blocks NO production in the obstructed renal medulla.

Nitric oxide has several important functions in the kidney [39–40]. In obstruction the role of endogenous nitric oxide, an endothelial-derivated relaxing factor (EDRF), in the regulation of renal hemodynamics has been previously investigated by Chevalier, Thornhill and Gomez [41].

Our present results show that endogenous NO participates in the vacuolar proton ATPase inhibition in unilateral obstruction. With regards to regulation of the enzyme by NO, vacuolar H^+-ATPase from synaptic vesicles of rat brain has recently been demonstrated to be inhibited by NO with a mechanism that involves S-nitrosylation of critical sulfhydryl groups of the enzyme [42]. The possibility that cGMP also regulates the H^+-ATPase activity via an indirect signaling pathway in the cells has been suggested previously [20].

Increased expression of angiotensin II mRNA has been demonstrated in the early phase of unilateral obstruction. The presence of the angiotensin II type 1 (AT_1) receptor in the distal tubule, including medullary collecting ducts, has been supported by both biochemical and molecular biological studies [8]. The AT_1 receptor transduces much of the biological effects of angiotensin.

Our study shows that H^+-ATPase activity was not inhibited when the obstructed IMCD was incubated with losartan, an antagonist of AT_1 receptor. When the concentrations of losartan were decreased from 10^{-5} to 10^{-8} mol/L, there was a corresponding gradual increase in H^+-ATPase activity, and the maximum recovery of the enzyme activity was observed at the concentration of 10^{-8} mol/L losartan.

Previously, Tojo, Tisher, and Madsen showed that preincubation of control CCD segments with 10^{-8} to 10^{-5} mol/L angiotensin II had a specific dose-dependent inhibitory effect on the vacuolar H^+-ATPase, with a maximum inhibition at 10^{-8} mol/L angiotensin II [22].

Because of the increased angiotensin converting enzyme (ACE) activity and despite a very low amount of renin mRNA, nonsuppressed angiotensin II levels were found in unilaterally obstructed ClKs in relation to CKs by El-Dahr et al [43]. Although our results in renal medulla homogenates from ClKs showed a small decrease in the calcium/calmodulin-independent and -dependent NOS activity induced by 10^{-5} to 10^{-8} mol/L losartan, we cannot suggest that there is an interaction between endogenous nitric oxide and angiotensin II through its AT_1 receptor in the contralateral kidney.

Our results do suggest that, through its AT_1 receptor, angiotensin II is involved in the vacuolar proton ATPase inhibition in obstruction.

No effect of the angiotensin II type 2 (AT_2) receptor on the inhibition of H^+-ATPase activity has been previously reported [22].

Because of the recovery of the proton ATPase activity to near control values after the incubation of obstructed IMCD in the presence of losartan in doses from 10^{-5} to 10^{-8} mol/L, and the previously mentioned results of an increase on the proton ATPase activity of obstructed IMCD in the presence of L-NAME and aminoguanidine, we looked for the relationship between endogenous NO and angiotensin II on the regulation of the enzyme activity.

A decrease in calcium/calmodulin-independent activity (iNOS) was demonstrated in the obstructed renal

medulla incubated with 10^{-5} to 10^{-8} mol/L losartan, the same losartan concentrations that showed a recovery of vacuolar H^+-ATPase activity. Similarly, lower values of cNOS activity were shown after the incubation of the obstructed renal medulla in the presence of decreasing concentrations of the AT_1 receptor antagonist (10^{-5} to 10^{-8} mol/L losartan).

The comparison between the calcium/calmodulin-independent NOS activity (iNOS) in renal medulla of OKs before and after incubation with losartan showed a more important decrease in iNOS activity than the one related to cNOS activity.

In agreement with these latter results, a dose dependent decrease in the nitrite generation was also demonstrated after incubating the obstructed renal medulla with decreasing concentrations of losartan, from 10^{-5} to 10^{-8} mol/L, with the lowest dose (10^{-8} mol/L) inducing the greatest decrease in nitrite.

According to our results, an interaction between the vasoconstrictor peptide angiotensin II through it AT_1 receptor and the endogenous vasodilator nitric oxide at the level of H^+-ATPase in the obstructed IMCD segments was demonstrated.

Thus, angiotensin II and endogenous nitric oxide influence each other's functions in unilateral ureteral obstruction.

Augmented glomerular cGMP generation after the stimulation of soluble guanylyl cyclase has been described previously in ipsilateral unilateral obstruction as a counterbalancing vasodilatory response modulated by AT_1 receptors to the angiotensin II-mediated vasoconstriction [44].

The mechanisms involved in the abolishment of the inhibitory effect of H^+-ATPase activity in obstruction and in the decrease generation of endogenous nitric oxide by the antagonist of AT_1 receptor of angiotensin II cannot be inferred from this study. The most likely explanation could involve the ability of angiotensin II to activate multiple signaling pathways. It remains to be established whether one or more signaling pathways are involved in the effect of AT_1 receptor antagonist on the proton ATPase activity in obstruction. It has been demonstrated that cGMP might serve as a second messenger to inhibit the proton ATPase in normal CCD [20]. Recently, it was shown in rat proximal tubules that angiotensin II signaling mediated by AT_1 receptors activates the NO-cGMP pathway. The angiotensin II and NO-cGMP signaling pathways appear to intersect at the level of intracellular calcium [45]. Moreover, evidence supporting a role for intracellular calcium [$(Ca^{2+})_i$] as a second messenger also exists. The rise in $(Ca^{2+})_i$ elicited by angiotensin II in the rat proximal tubule has been demonstrated to be mediated by the AT_1 receptor subtype. Results of a recent study suggest that intracellular modulation of proton secretion in IMCDs requires changes in $(Ca^{2+})_i$ [46].

In renal epithelial cells, the mechanisms that trigger the increases of $(Ca^{2+})_i$ and NO during cell injury involve the activation of G-protein–coupled receptors for substances such as angiotensin II, thromboxane, and thrombin. Activation of a G protein, $G\alpha_{13}$, specifically induces expression of iNOS in renal epithelial cells, demonstrating that G-protein–dependent signaling systems are capable of inducing iNOS and, hence, an increase in NO levels [47].

The expression of guanosine 5-triphosphate (GTP)-binding protein (G proteins) associated with H^+-ATPase activity in intercalated cells of the rat medulla with a functional up-regulation of the pump has been reported. It has been suggested that the H^+-ATPase can be directly regulated by a G protein or indirectly via an intermediate second messenger, such as adenosine 3-5'-cyclic monophosphate, although the particular G protein involved remains to be identified [48].

Because of these results, we conclude that endogenous NO, while mainly increased by iNOS, is involved in the inhibition of H^+-ATPase activity of IMCD segments. The recovery of H^+-ATPase activity in IMCD segments of obstructed kidneys induced by losartan could be related to a decrease of the inducible nitric oxide synthase activity.

ACKNOWLEDGMENTS

This work was performed with financial support from the Research and Technology Council of Cuyo University (CIUNC) from Mendoza, Argentina, to P.G.V. Portions of this study were published in an abstract form (*Pediatr Nephrol* 12:C95, 1998). Portions of this study were presented as an abstract form at the XVth International Congress of Nephrology in Buenos Aires, Argentina, May 2–6, 1999. Losartan, code MP-L-459/4, was a gift from Roemmers Laboratory.

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